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The impact of fruit fly gut bacteria on the rearing of the parasitic wasp *Diachasmimorpha longicaudata*

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Abstract

Area-wide integrated pest management strategies against tephritid fruit flies include the release of fruit fly parasitic wasps in the target area. Mass rearing of parasitic wasps is essential for the efficient application of biological control strategies. Enhancement of fruit fly host fitness through manipulation of their gut-associated symbionts might also enhance the fitness of the produced parasitic wasps and improve the parasitoid rearing system. In the current study, we added three gut bacterial isolates originating from *Ceratitis capitata* (Wiedemann) and four originating from *Bactrocera oleae* (Rossi) (both Diptera: Tephritidae) to the larval diet of *C. capitata* and used the bacteria-fed larvae as hosts for the development of the parasitic wasp *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae). We evaluated the effect of the bacteria on wasp life-history traits and assessed their potential use for the improvement of *D. longicaudata* rearing. *Enterobacter* sp. AA26 increased fecundity and parasitism rate and accelerated parasitoid emergence. *Providencia* sp. AA31 led to faster emergence of both male and female parasitoids, whereas *Providencia* sp. 22 increased the production of female progeny. *Bacillus* sp. 139 increased parasitoid fecundity, parasitism rate, and production of female progeny. *Serratia* sp. 49 accelerated parasitoid emergence for both males and females and increased production of female progeny. *Klebsiella oxytoca* delayed parasitoid emergence and *Enterobacter* sp. 23 decreased parasitoid fecundity and parasitism rate. Our findings demonstrate a wide range of effects of fruit fly gut symbionts on parasitoid production and reveal a great potential of bacteria use towards enhancement of parasitic wasp rearing.

Introduction

Tephritid fruit flies (Diptera) belonging to the genera *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, *Rhagoletis*, and *Zeugodacus* have a destructive impact on fruit orchards and are considered serious agricultural pests worldwide (Bateman, 1972; White & Elson-Harris, 1992; Vargas et al., 2015; Doorenweerd et al., 2018). Due to their economic importance, fruit flies have been considered targets for area-wide integrated pest management (IPM) strategies that include the combination of augmentative biological control along with other suppression techniques such as the sterile insect technique (SIT), ground or aerial bait spraying, fruit stripping, and mass trapping. Augmentative biological control is an environment-friendly strategy for pest population suppression that depends on the mass release of natural enemies, such as parasitic wasps, in the target area (Knipling, 1992). It has already been applied towards population suppression of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Wong et al., 1991; Vargas et al., 2001), *Bactrocera* spp. (Vargas et al., 2004; Harris et al., 2010), and *Anastrepha* spp. (Sivinski et al., 1996; Montoya et al., 2000).

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Diachasmimorpha longicaudata (Ashmead) (Hymenoptera: Braconidae) is a solitary koinobiont endoparasitoid wasp that lays its eggs inside fruit fly larvae, where they complete their development through to the adult stage (Greany et al., 1976). It is considered one of the most significant biological control agents for augmentative applications against economically important Tephritidae fruit flies. It has already been used towards the population control of *C. capitata* (Wong et al., 1991; Sánchez et al., 2016), *Bactrocera dorsalis* (Hendel) (Vargas et al., 2012), *Anastrepha obliqua* (Macquart), and *Anastrepha ludens* (Loew) (Montoya et al., 2000), and has also been suggested as potential biological control agent of the olive fruit fly, *Bactrocera oleae* (Rossi) (Sime et al., 2006, 2008).

Bactrocera oleae, the most serious threat of olive fruits and olive oil production in the Mediterranean region, South and Central Africa, Canary Islands, Near and Middle East, California (USA), and Central America (Rice et al., 2003; Copeland et al., 2004; Augustinos et al., 2005; Nardi et al., 2005; Invasive Species Compendium, 2020), has been suggested as a potential target of integrated SIT programs combined with parasitoid releases (Nestel et al., 2016). Potential use of parasitoid wasps for the biological control of the olive fruit fly has been assessed by recent studies suggesting the utilization of a range of parasitic wasps such as *Bracon celer* Szépligeti, *Psyttalia humilis* (Silvestri), *Psyttalia lounsburyi* (Silvestri), *Psyttalia ponerophaga* (Silvestri), *Utetes africanus* (Silvestri) (Daane & Johnson, 2010; Daane et al., 2015), *Fopius arisanus* (Sonan), *Diachasmimorpha kraussii* (Fullaway), and *D. longicaudata* (Sime et al., 2006, 2008). Several studies have attempted to perform classical (augmentative) biological control of *B. oleae* but their success was limited for several reasons, such as difficulties in transportation of the parasitoids to the field, low performance of the released parasitoids in the field due to the reversal of seasons in the Northern and Southern Hemispheres, and problems with mass rearing of both the olive fruit fly and the parasitoids (Bartlett & Clausen, 1978). One of the most crucial steps in large-scale applications of biological control is the efficient mass rearing of great numbers of robust parasitoid wasps (van Lenteren, 2000). Rearing of parasitoids on olive fruit fly larvae is currently inefficient due to the high cost of the host rearing system. Alternative hosts such as the closely related Mediterranean fruit fly have been used in the rearing of *P. concolor*, *P. humilis*, and *D. longicaudata* (Ovruski et al., 2011; Yokoyama et al., 2012; Daane et al., 2015). In addition to host suitability, other parasitoid mass-rearing challenges are related to several life-history aspects, such as female fecundity, adult size and longevity, progeny sex ratio, and parasitism rate of the wasps (Messing et al., 1993; Eben et al., 2000; Yokoyama et al., 2012).

Several studies on tephritid fruit flies found that symbiotic microbes have beneficial effects on various functions of their insect hosts. These functions include nutrition and metabolism (Behar et al., 2005; Bourtzis & Miller, 2008; Ben-Yosef et al., 2014), reproduction (Ben-Yosef et al., 2008), oviposition (Jose et al., 2019), foraging behavior (Akami et al., 2019), detoxification processes and insecticide resistance (Cheng et al., 2017; Guo et al., 2017), and the insect reaction to the plant defense mechanisms against its larval development (Ben-Yosef et al., 2015). Additionally, it has been shown that the incorporation of gut bacteria in larval or adult artificial diets positively affects life-history traits related to artificial rearing such as pupal weight, adult size, survival, mating competitiveness, flight ability, immature development duration, female fecundity, and oviposition behavior (Niyazi et al., 2004; Behar et al., 2008; Meats et al., 2009; Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Khan et al., 2014; Sacchetti et al., 2014; Rull et al., 2015; Augustinos et al., 2015; Kyritsis et al., 2017; Khaeso et al., 2018; Jose et al., 2019).

Similar to the fruit flies, symbionts might also enhance the fitness of the parasitoids produced. Chiel et al. (2009) investigated the transmission of the bacterial symbionts *Rickettsia* and *Hamiltonella* from their host, the sweet potato whitefly, *Bemisia tabaci* (Gennadius), to three species of whitefly parasitoids. They found that microbe horizontal transmission from the bacteria-infected whitefly to the parasitic wasp through feeding is possible and might vary for various bacteria species and parasitoids. Therefore, similar to the whitefly example, we hypothesized that feeding the fruit fly larvae with diet enriched with beneficial bacteria and offering them as hosts to the parasitic wasps would lead to the acquisition of the bacteria by the parasitoid offspring during their development inside the infected larvae. This could have beneficial effects on parasitoid fitness similar to the positive effects of the gut symbionts on their fruit fly host.

In the current study, we tested this hypothesis by feeding *C. capitata* larvae with bacteria-enriched larval diets, using seven bacterial isolates originating from both the *B. oleae* and *C. capitata* digestive systems. We used the bacteria-fed larvae as hosts for the development of *D. longicaudata* and evaluated the effect of the bacteria on life-history traits of the wasp that are important for efficient parasitoid rearing. Although we used bacterial species isolated from both *C. capitata* and *B. oleae*, we could only perform our tests with medfly larvae as hosts for the parasitoid development, because difficulties with the current rearing system of the olive fruit fly prevent production of sufficient larvae for such experiments.

Materials and methods

Diachasmimorpha longicaudata and *Ceratitis capitata* rearing conditions

The *D. longicaudata* strain, kindly provided by Dr. Francisco Beitia of the IVIA (Spain), was maintained under constant environmental conditions at 25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod. *Diachasmimorpha longicaudata* adults were kept in plexiglass cages (50 × 40 × 40 cm) with a round opening (15 cm) covered with fine mesh at the top and were constantly provided with water and honey. *Diachasmimorpha longicaudata* rearing was done on *C. capitata* larvae. Third instar *C. capitata* were removed from the larval diet, irradiated at 40 Gy (standard procedure to prevent the emergence of adult fruit flies from any non-parasitized pupa; Cancino et al., 2012) and placed in Petri dishes (15 cm diameter) with an opening of approximately 10 cm diameter in the center of their lids which was covered with a fine mesh screen. The larvae were placed on the lid and covered with a moistened sponge of the same diameter as the lid. The sponge was subsequently covered with a piece of plexiglass of the same diameter to keep the sponge and the larvae firmly inside the Petri dish and create the oviposition unit containing the larvae hosts in which *D. longicaudata* females would lay their eggs. Larvae were placed at the top of the adult parasitoid cage 8–10 days after parasitoid emergence to allow *D. longicaudata* egg-laying. Parasitoid oviposition in the available larvae was facilitated by placing the Petri dish with the fruit fly larvae (oviposition unit) at the top of the adult cage (on the site of the 10-cm-diameter round opening), with the lid side facing down. Four h after exposition, the larvae were transferred into a plastic box with sawdust to allow pupation. All procedures took place under controlled temperature, humidity, and light conditions (25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod).

Ceratitis capitata strain 'Tucuman', kindly provided by Dr. Teresa Vera, INTA Castelar, Argentina, originated from Tucuman (Argentina) and was maintained at the same environmental conditions with constant provision of water and adult diet consisting of sugar and yeast hydrolyzate at a 3:1 ratio (Caceres, 2002).

Origin and characterization of gut bacteria

Enterobacter sp. AA26 and *Providencia* sp. AA31 strains used in this study were previously isolated from *C. capitata* (Augustinos et al., 2015). The *Klebsiella oxytoca* strain was kindly provided by Prof. Edouard Jurkevitch of the Hebrew University of Jerusalem (Rehovot, Israel) and has been used in previous studies (Behar et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Kyritsis et al., 2017). *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 were previously isolated from wild olive fruit flies, kindly provided by Mr. Jaime García de Oteyza of TRAGSA (Spain) (Koskinioti et al., 2020). All bacterial strains were revived from glycerol stocks kept at −80 °C by streaking on Luria-Bertani (LB) agar medium plates. Single bacterial colonies were selected and inoculated in LB broth medium for subsequent rearing experiments.

Bacterial enrichment of *Ceratitis capitata* larval diet

The revived cultures were added to the standard wheat bran-based medfly larval diet (Hooper, 1987) in a titer of 10^8 bacteria per g of diet. The titer for each bacterial isolate was determined by measuring the optical density (OD) of each culture. The OD required to reach the appropriate titer for each isolate was determined by bacterial colony counting of serial dilutions of an initial culture with known OD. Bacterial cultures with the appropriate OD were centrifuged and resuspended in 20 ml LB medium, before mixing with 1 kg of larval diet. The same number of autoclaved (dead) bacteria was incorporated in the diet to test whether live bacteria have an effect through interaction with the host larvae and subsequently the parasitoid, or whether they only serve as a nutrient source. The control treatment consisted of the standard wheat bran-based larval diet mixed with 20 ml of LB medium (without bacteria). The diet was prepared by hand mixing directly before the addition of the eggs.

Ceratitis capitata egg collection and transfer to bacteria-enriched diets

Eggs laid during a period of 8 h were collected from 8-day-old *C. capitata* females and placed on moist filter paper resting on wet sponges infused with water. Twenty-four h after egg collection, filter papers with 300 eggs each were transferred to a Petri dish (70 × 15 mm) with 150 g wheat bran diet. Three replicates of 300 eggs were used for each treatment (live and autoclaved bacteria for each strain and the control treatment). After their transfer, the eggs were incubated under constant environmental conditions at 25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod. Third instars (10 days old) were separated from the diet, irradiated at 40 Gy (to prevent the emergence of adult fruit flies from any non-parasitized pupae) and used as hosts for rearing of *D. longicaudata* for subsequent experiments.

Exposure of the host larvae to *Diachasmimorpha longicaudata*

Three cages (replicates) of five *D. longicaudata* couples were prepared for each treatment (live and autoclaved bacteria for each isolate and the control treatment). Eight days after their emergence, the mated female parasitoids were allowed to parasitize third instar *C. capitata* that were fed

on the bacteria-enriched diets. One hundred larvae were offered to each cage of five *D. longicaudata* couples (20 larvae per female) for 12 h. After the 12-h exposure, larvae were removed and allowed to pupate in plastic boxes containing sawdust. Pupae were counted for each replicate and allowed to develop under controlled conditions (25 ± 1 °C, 60 ± 5% r.h., and L14:D10 photoperiod) until emergence of adult parasitoids.

Effect of bacteria-enriched host larval diet on *Diachasmimorpha longicaudata* life-history traits

The number and sex of the emerged parasitoids were recorded for each replicate of each treatment. Parasitoid fecundity was determined as the total number of the parasitoids that emerged divided by the number of parasitoid females that were alive on the day of oviposition in each replicate. The parasitism rate was calculated by dividing the total number of emerged parasitoids by the number of fly larvae that pupated in each replicate. Sex ratio was calculated by dividing the number of emerged *D. longicaudata* females by the total number of emerged offspring for each replicate. Daughter production per individual female was calculated by multiplying the fraction of females (no. emerged females/total no. emerged parasitoids) by fecundity. Egg-to-adult developmental duration was determined by recording the number and sex of emerged parasitoids every day.

Statistical analysis

The effects of the various bacteria treatments on fecundity and female progeny production were estimated with general linear models with 'treatment' as the independent variable. Levene's test was performed to test for homogeneity of variances of raw data. A post-hoc test was used for multiple comparisons of the tested groups with Bonferroni adjustment. The effect of added bacteria on parasitism rate and sex ratio was determined by binary logistic regression analysis (BLR) with Bonferroni correction to adjust the significance threshold for multiple comparisons. The Kaplan-Meier test was used to determine the effect of added bacteria on the egg-to-adult developmental duration of the parasitic wasp. Pairwise comparisons among treatments were tested with the Mantel-Cox log-rank test corrected for multiple comparisons with a threshold of $\alpha = 0.003$. All datasets were analyzed in IBM SPSS v.24.0 (IBM, Armonk, NY, USA).

Results

Effect of bacteria-enriched host larval diet on parasitoid fecundity

Diachasmimorpha longicaudata fecundity was affected by bacteria treatment ($F_{14,30} = 26.280$, $P<0.001$; Table 1). More specifically, both live and dead *Enterobacter* sp. AA26 treatments increased wasp fecundity compared to the control treatment ($P<0.001$ and 0.003, respectively). No difference was detected between the effect of live and dead *Enterobacter* sp. AA26 on fecundity ($P = 1$; Figure 1

). *Providencia* sp. AA31 and *K. oxytoca* (both live and dead) had no effect on parasitoid fecundity. On the other hand, live *Enterobacter* sp. 23 decreased parasitoid fecundity compared to both the control and dead *Enterobacter* sp. 23 treatment ($P<0.001$), whereas *Providencia* sp. 22 (both live and dead) had no significant effect. Live treatment of *Bacillus* sp. 139 increased fecundity compared to the control ($P = 0.035$). Dead *Bacillus* sp. 139 and *Serratia* sp. 49 (both live and dead) had no effect on parasitoid fecundity (Figure 1, Table 1).

Effects of bacteria-enriched host larval diet on parasitism rate

Diachasmimorpha longicaudata parasitism rate was affected by bacterial enrichment of host larval diet (overall Wald's test: $\chi^2 = 254.427$, d.f. = 14, $P<0.001$; Table S2). *Enterobacter* sp. AA26 increased parasitism rate in both live and dead bacteria treatments compared to the control ($P<0.001$; Figure 2

, Table 2). Comparison between live and dead *Enterobacter* sp. AA26 indicated no difference between the two treatments (Table 2). *Providencia* sp. AA31 and *K. oxytoca* (both live and dead) had no effect on parasitism rate compared to the control. Live *Enterobacter* sp. 23 decreased parasitism rate compared to both the control and the dead treatment ($P<0.001$) No difference was detected between the control and dead *Enterobacter* sp. 23 treatment. *Bacillus* sp. 139 increased parasitism rate in the live treatment compared to control ($P = 0.036$) but had no effect in the dead treatment ($P = 1$). *Providencia* sp. 22 and *Serratia* sp. 49 (both live and dead) had no effect on *D. longicaudata* parasitism rate compared to control (Figure 2, Table 2).

Effects of bacteria-enriched host larval diet on parasitoid sex ratio

Diachasmimorpha longicaudata sex ratio was not affected by treatment as indicated by the binary logistic regression model for all the bacteria treatments (overall Wald's test: $\chi^2 = 21.128$, d.f. = 14, $P = 0.098$). However, pairwise comparisons revealed an increase of female fraction in live *Providencia* sp. 22 treatment compared to the control ($P = 0.022$; Figure 3, Table 3), but dead *Providencia* sp. 22 had no effect ($P = 0.07$). No difference was observed between the live and dead treatment of *Providencia* sp. 22 ($P = 0.66$). Similarly, live *Bacillus* sp. 139 increased the fraction of *D. longicaudata* female progeny compared to the control ($P = 0.021$), whereas dead *Bacillus* sp. 139 had

Table 1 Effect of the provision of bacteria in the host larval diet on *Diachasmimorpha longicaudata* fecundity (no. emerged parasitoids per female). Pairwise comparisons, first row of each cell: mean difference, second row: Bonferroni adjusted P

GLM corrected overall model		$F_{14,30} = 26.280$, adjusted P<0.001													
Pairwise comparisons															
		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Mean fecundity	13.13	16.27	15.73	12.00	12.93	13.53	12.47	8.47	11.93	14.87	13.60	15.27	14.53	12.73	12.80
SEM	0.437	0.24	0.133	0.231	0.406	0.372	0.240	0.371	0.267	0.352	0.306	0.437	0.533	0.593	0.30

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	−3.133														
	<0.001														
Enterobacter AA26 Dead	−2.600	0.533													
	0.003	1.0													
Providencia AA31 Live	1.133	4.267	3.733												
	1.0	<0.001	<0.001												
Providencia AA31 Dead	0.200	3.333	2.800	−0.933											
	1.0	<0.001	0.001	1.0											
K. oxytoca Live	−0.400	2.733	2.200	−1.533	−0.600										
	1.0	0.001	0.025	0.706	1.0										
K. oxytoca Dead	0.667	3.800	3.267	−0.467	0.467	1.067									
	1.0	<0.001	<0.001	1.0	1.0	1.0									
Enterobacter 23 Live	4.667	7.800	7.267	3.533	4.467	5.067	4.000								
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001								
Enterobacter 23 Dead	1.200	4.333	3.800	0.067	1.0	1.600	0.533	−3.467							
	1.0	<0.001	<0.001	1.0	1.0	0.514	1.0	<0.001							
Providencia 22 Live	−1.733	1.400	0.867	−2.867	−1.933	−1.333	−2.400	−6.400	−2.933						
	0.268	1.0	1.0	0.001	0.098	1.0	0.009	<0.001	<0.001						
Providencia 22 Dead	−0.467	2.667	2.133	−1.600	−0.667	−0.067	−1.133	−5.133	−1.667	1.267					
	1.0	0.002	0.035	0.514	1.0	1.0	1.0	<0.001	0.372	1.0					
Bacillus 139 Live	−2.133	1.0	0.467	−3.267	−2.333	−1.733	−2.800	−6.800	−3.333	−0.400	−1.667				
	0.035	1.0	1.0	<0.001	0.012	0.268	0.001	<0.001	<0.001	1.0	0.372				
Bacillus 139 Dead	−1.400	1.733	1.200	−2.533	−1.600	−1.0	−2.067	−6.067	−2.600	0.333	−0.933	0.733			
	1.0	0.268	1.0	0.004	0.514	1.0	0.049	<0.001	0.003	1.0	1.0	1.0			
Serratia 49 Live	0.400	3.533	3.000	−0.733	0.200	0.800	−0.267	−4.267	−0.800	2.133	0.867	2.533	1.800		
	1.0	<0.001	<0.001	1.0	1.0	1.0	1.0	<0.001	1.0	0.035	1.0	0.004	0.193		
Serratia 49 Dead	0.333	3.467	2.933	−0.800	0.133	0.733	−0.333	−4.333	−0.867	2.067	0.800	2.467	1.733	−0.067	0.067
	1.0	<0.001	<0.001	1.0	1.0	1.0	1.0	<0.001	1.0	0.049	1.0	0.006	0.268	1.0	1.0

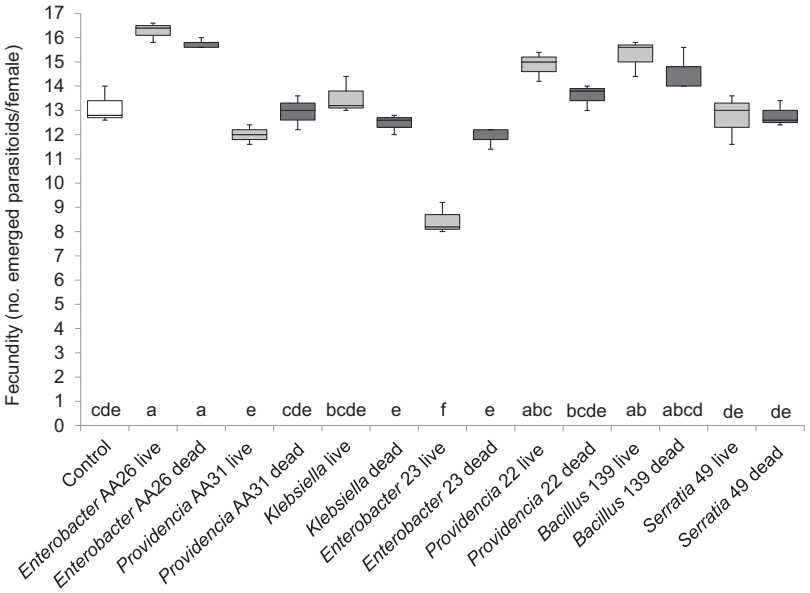

Figure 1 Effect of medfly larval diets enriched with LB medium (without bacteria; control), *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on *Diachasmimorpha longicaudata* fecundity (no. emerged parasitoids produced per female). The top and bottom of the boxes represent the 25th and 75th percentiles, indicating the inter-quartile range. The horizontal line within the box represents the median value. The whiskers indicate the highest and lowest observations and define the variability outside the inter-quartile range. Treatments marked with different letters on the x-axis cause a significant difference in parasitoid fecundity (GLM: $P<0.05$).

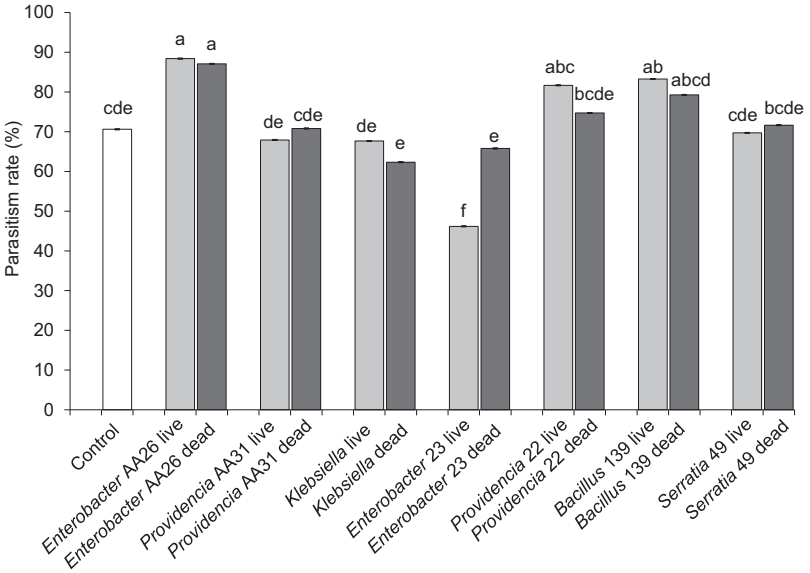

Figure 2 Effect of medfly larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (± SEM) *Diachasmimorpha longicaudata* parasitism rate (%; 100 × no. emerged parasitoids/no. pupated fly larvae). Means capped with different letters are significantly different (BLR: $P<0.05$)

no effect ($P = 0.17$). No difference was observed between the live and dead treatment of *Bacillus* sp. 139 ($P = 0.36$). Live *Serratia* sp. 49 also affected sex ratio ($P = 0.026$) compared to the control, but dead *Serratia* sp. 49 treatment did not ($P = 0.062$). No difference was observed between the live and dead treatment of *Serratia* sp. 49

Table 2 Effect of the provision of bacteria in the host larval diet on *Diachasmimorpha longicaudata* parasitism rate (no. emerged parasitoids/no. pupated larvae). Pairwise comparisons, first row of each cell: mean difference, second row: Bonferroni adjusted P

Overall test results		Wald χ^2 = 26.280, d.f. = 14, P <0.001													
Pairwise comparisons															
		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Mean parasitism	70.58	88.40	87.09	67.96	70.77	74.34	68.49	46.15	65.80	81.86	74.71	83.24	79.25	69.64	71.63
SEM	1.58	0.79	0.69	1.76	1.32	1.53	0.55	1.40	0.66	3.76	0.85	1.19	2.36	1.87	0.62

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	−0.18														
	<0.001														
Ente*robacter* AA26 Dead	−0.16	0.01													
	<0.001	1.0													
Providencia AA31 Live	0.03	0.20	0.19												
	1.0	<0.001	<0.001												
P*rovidencia* AA31 Dead	0.00	0.18	0.16	−0.03											
	1.0	<0.001	<0.001	1.0											
K. oxytoca Live	0.03	0.21	0.19	0.00	0.03										
	1.0	0.001	<0.001	1.0	1.0										
K. oxytoca Dead	0.08	0.26	0.25	0.06	0.08	0.05									
	1.0	<0.001	<0.001	1.0	1.0	1.0									
Enterobacter 23 Live	0.24	0.42	0.41	0.22	0.25	0.21	0.16								
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.009								
Enterobacter 23 Dead	0.05	0.23	0.21	0.02	0.05	0.02	−0.03	−0.20							
	1.0	<0.001	<0.001	1.0	1.0	1.0	1.0	<0.001							
Providencia 22 Live	−0.11	0.07	0.05	−0.14	−0.11	−0.14	−0.19	−0.36	−0.16						
	0.216	1.0	1.0	0.021	0.270	0.009	<0.001	<0.001	0.002						
Providencia 22 Dead	−0.04	0.14	0.12	−0.07	−0.04	−0.07	−0.12	−0.29	−0.09	0.07					
	1.0	0.003	0.021	1.0	1.0	1.0	0.131	<0.001	1.0	1.0					
Bacillus 139 Live	−0.13	0.05	0.04	−0.15	−0.12	−0.16	−0.21	−0.37	−0.17	−0.02	−0.09				
	0.036	1.0	1.0	0.003	0.047	0.001	<0.001	<0.001	<0.001	1.0	1.0				
Bacillus 139 Dead	−0.09	0.09	0.08	−0.11	−0.08	−0.12	−0.17	−0.33	−0.13	0.02	−0.05	0.04			
	1.0	0.351	1.0	0.273	1.0	0.151	0.001	<0.001	0.038	1.0	1.0	1.0			
Serratia 49 Live	0.01	0.19	0.17	−0.02	0.01	−0.02	−0.07	−0.24	−0.04	0.12	0.05	0.14	0.10		
	1.0	<0.001	<0.001	1.0	1.0	1.0	1.0	<0.001	1.0	0.102	1.0	0.015	1.0		
Serratia 49 Dead	−0.01	0.17	0.15	−0.04	−0.01	−0.04	−0.09	−0.25	−0.06	0.10	0.03	0.12	0.08	−0.02	0.02
	1.0	<0.001	0.001	1.0	1.0	1.0	1.0	<0.001	1.0	0.102	1.0	0.113	1.0	1.0	1.0

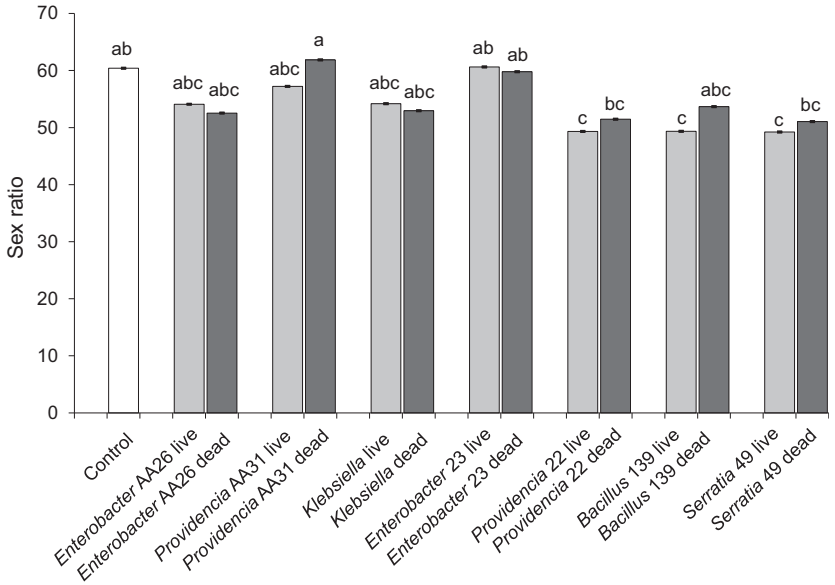

Figure 3 Effect of medfly larval diets enriched with *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on mean (± SEM) *Diachasmimorpha longicaudata* sex ratio (%; 100 × no. females/total no. emerged parasitoids). Means capped with different letters are significantly different (BLR: $P<0.05$)

($P = 0.72$). *Enterobacter* sp. AA26, *Providencia* sp. AA31, *K. oxytoca*, and *Enterobacter* sp. 23 did not affect *D. longicaudata* sex ratios ($P>0.05$; Figure 3, Table 3).

Effects of bacteria-enriched host larval diet on female progeny production

Diachasmimorpha longicaudata female production was affected by treatment as indicated by the binary logistic regression model for all the bacteria treatments (overall Wald's test: $\chi^2 = 672.384$, d.f. = 14, $P<0.001$). *Enterobacter* sp. AA26 increased female production in both live and dead bacteria treatments compared to the control ($P<0.001$; Figure 4, Table 4). *Providencia* sp. AA31 (both live and dead) had no effect on female production compared to the control ($P>0.05$). Live *K. oxytoca* increased female production compared to the control ($P<0.05$) whereas they had no effect when dead ($P>0.05$). Live *Enterobacter* sp. 23 decreased female production compared to both the control and the treatment with dead *Enterobacter* sp. 23 ($P<0.001$). No difference was detected between the control and dead *Enterobacter* sp. 23 treatment ($P>0.05$). *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 (both live and dead) increased the production of female progeny per female compared to the control ($P<0.05$). Live treatments of *Providencia* sp. 22 and *Bacillus* sp. 139 also increased female production compared to the respective dead treatments ($P<0.05$; Figure 4, Table 4).

Effects of bacteria-enriched host larval diet on parasitoid egg-to-adult developmental duration

Developmental duration from the day of parasitization to the day of parasitoid emergence was affected by the provision of live and dead *Enterobacter* sp. AA26, *Providencia* sp. AA31, *K. oxytoca*, and *Serratia* sp. 49, in both males and females (Tables 5 and 6). More specifically, *Enterobacter* sp. AA26 accelerated parasitoid emergence of both males (live: log-rank test $\chi^2 = 52.754$; dead: $\chi^2 = 47.312$) and females (live: $\chi^2 = 73.754$; dead: $\chi^2 = 70.643$, all $P<0.001$; Figure 5, Tables 5 and 6), but there was no differential effect of the live and the dead treatment (males: $\chi^2 = 0.065$, $P = 0.80$; females: $\chi^2 = 0.354$, $P = 0.55$). Similarly, *Providencia* sp. AA31 led to faster emergence of both males (live: $\chi^2 = 38.220$; dead: $\chi^2 = 31.271$) and females (live: $\chi^2 = 55.491$; dead: $\chi^2 = 52.318$, all $P<0.001$) with no difference between the live and autoclaved treatment (males: $\chi^2 = 0.970$, $P = 0.33$; females: $\chi^2 = 0.228$, $P = 0.63$). Immature development was delayed by *K. oxytoca* in both males (live: $\chi^2 = 15.668$; dead: $\chi^2 = 17.128$) and females (live: $\chi^2 = 31.324$; dead: $\chi^2 = 29.526$, all $P<0.001$), again with no difference between live and dead treatment ($P>0.05$). *Serratia* sp. 49 also led to faster development of both males (live: $\chi^2 = 38.220$; dead: $\chi^2 = 31.271$) and females (live: $\chi^2 = 55.491$; dead: $\chi^2 = 52.318$, all $P<0.001$), and there was no difference between live and dead treatment. *Enterobacter* sp. 23, *Providencia* sp. 22, and *Bacillus* sp. 139 had no effect on

Table 3 Effect of the provision of bacteria in the host larval diet on the sex ratio of the emerged *Diachasmimorpha longicaudata* parasitoids. Pairwise comparisons, first row of each cell: mean difference, second row: Bonferroni adjusted P

Overall test results		Wald χ^2 = 21.128, d.f. = 14, P = 0.098													
Pairwise comparisons															
		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	−0.06														
	0.182														
Enterobacter AA26 Dead	−0.08	−0.02													
	0.099	0.733													
Providencia AA31 Live	−0.03	0.03	0.05												
	0.530	0.522	0.341												
Providencia AA31 Dead	0.01	0.08	0.09	0.05											
	0.769	0.101	0.051	0.361											
K. oxytoca Live	−0.06	0.00	0.02	−0.03	−0.08										
	0.208	0.985	0.730	0.550	0.120										
K. oxytoca Dead	−0.07	−0.01	0.00	−0.04	−0.09	−0.01									
	0.139	0.811	0.935	0.409	0.077	0.805									
Enterobacter 23 Live	0.00	0.07	0.08	0.03	−0.01	0.06	0.08								
	0.968	0.225	0.136	0.549	0.826	0.247	0.175								
Enterobacter 23 Dead	−0.01	0.06	0.07	0.03	−0.02	0.06	0.07	−0.01							
	0.901	0.243	0.140	0.623	0.681	0.270	0.186	0.881							
Providencia 22 Live	−0.11	−0.05	−0.03	−0.08	−0.13	−0.05	−0.04	−0.11	−0.10						
	0.022	0.302	0.491	0.113	0.010	0.315	0.466	0.039	0.035						
Providencia 22 Dead	−0.09	−0.03	−0.01	−0.06	−0.10	−0.03	−0.01	−0.09	−0.08	0.02					
	0.070	0.579	0.822	0.258	0.036	0.583	0.771	0.100	0.101	0.658					
Bacillus 139 Live	−0.11	−0.05	−0.03	−0.08	−0.13	−0.05	−0.04	−0.11	−0.10	0.00	−0.02				
	0.021	0.301	0.490	0.112	0.009	0.314	0.465	0.038	0.035	0.997	0.659				
Bacillus 139 Dead	−0.07	0.00	0.01	−0.04	−0.08	−0.01	0.01	−0.07	−0.06	0.04	0.02	0.04			
	0.165	0.926	0.810	0.477	0.092	0.915	0.884	0.205	0.220	0.361	0.651	0.360			
Serratia 49 Live	−0.11	−0.05	−0.03	−0.08	−0.13	−0.05	−0.04	−0.11	−0.11	0.00	−0.02	0.00	−0.04		
	0.026	0.311	0.494	0.121	0.012	0.323	0.468	0.043	0.040	0.982	0.654	0.979	0.368		
Serratia 49 Dead	−0.09	−0.03	−0.02	−0.06	−0.11	−0.03	−0.02	−0.10	−0.09	0.02	0.00	0.02	−0.03	0.02	−0.02
	0.062	0.526	0.757	0.231	0.031	0.531	0.711	0.089	0.089	0.728	0.932	0.729	0.595	0.721	0.721

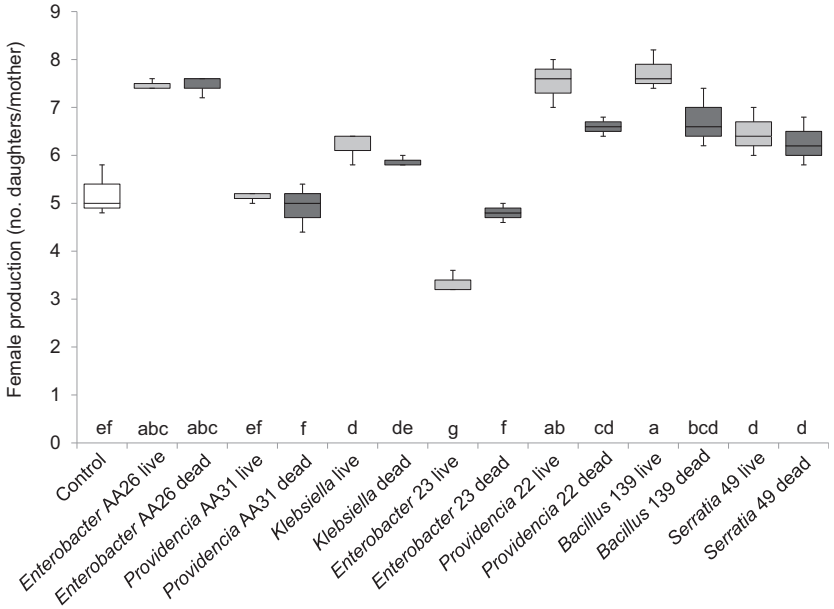

Figure 4 Effect of medfly larval diets enriched with LB medium (without bacteria; control), *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on *Diachasmimorpha longicaudata* female production (number of female offspring produced per female mother). The top and bottom of the boxes represent the 25th and 75th percentiles, indicating the inter-quartile range. The horizontal line within the box represents the median value. The whiskers indicate the highest and lowest observations and define the variability outside the inter-quartile range. Treatments marked with different letters on the x-axis cause a significant difference in parasitoid female production (GLM: $P<0.05$)

developmental duration of males and females ($P>0.003$; Figure 5, Tables 5 and 6).

Discussion

We assessed the effect of bacteria-fed medfly larvae on life-history traits of the parasitic wasp *D. longicaudata*. Our results demonstrated that *Enterobacter* sp. AA26 (live and autoclaved) increased fecundity, parasitism rate, and female production, accelerated emergence of both male and female parasitoids, and did not affect sex ratio of the emerged wasps. The positive effects of *Enterobacter* sp. AA26 on these life-history traits indicated that it might be used for faster and more productive laboratory rearing of *D. longicaudata*. *Enterobacter* sp. AA26 has also been tested as a larval diet additive in *C. capitata*, in which it increased pupal and adult productivity and induced faster development (Augustinos et al., 2015). *Enterobacter* sp. AA26 generally improves performance in the olive fruit fly as well, as it increases pupal weight, pupal and adult recovery, and reduces the egg-to-adult developmental time (Koskinioti et al., 2020). The results of these two studies in the medfly and the olive fruit fly, combined with the positive effect on *D. longicaudata* laboratory production, indicate that *Enterobacter* sp. AA26 could be used to improve the production of both the parasitic wasp and the fruit fly host. The effect of rearing *D. longicaudata* on *Enterobacter* sp. AA26-infected olive fruit fly larvae instead of medfly remains to be investigated.

Providencia sp. AA31 led to faster emergence in both male and female parasitoids (both live and autoclaved treatment) but had no significant effect on parasitoid fecundity, parasitism rate, offspring sex ratio, and female production. *Providencia* sp. AA31 also had an overall positive effect on the olive fruit fly laboratory rearing by increasing pupal and adult recovery (Koskinioti et al., 2020). The performance of *D. longicaudata* on *Providencia* sp. AA31-supplemented olive fruit fly larvae instead of medfly larvae remains yet to be investigated.

Treatment with live *Providencia* sp. 22 increased *D. longicaudata* offspring sex ratio and the production of female progeny per female, and had no significant effect on female fecundity, parasitism rate, and egg-to-adult developmental duration in males or females. Autoclaved *Providencia* sp. 22 increased the production of female progeny per female and caused no significant effect on any of the other studied life-history traits. The enhancement of female production by *Providencia* sp. 22 is of interest because females are responsible for both host-seeking and egg-laying; hence, increased numbers of female progeny are particularly

Table 4 Effect of the provision of bacteria in the host larval diet on *Diachasmimorpha longicaudata* female production. Pairwise comparisons, first row of each cell: mean difference, second row: Bonferroni adjusted P

GLM corrected overall model		$F_{14,30}$ = 672.384, adjusted P<0.001													
Pairwise comparisons															
		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Mean female production	5.20	7.47	7.47	5.13	4.93	6.20	5.87	3.33	4.80	7.53	6.60	7.73	6.73	6.47	6.27
SEM	0.306	0.067	0.133	0.067	0.291	0.200	0.067	0.133	0.115	0.291	0.115	0.240	0.353	0.291	0.291

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	−2.267														
	<0.001														
Enterobacter AA26 Dead	−2.267	0.000													
	<0.001	1.0													
Providencia AA31 Live	0.067	2.333	2.333												
	1.0	<0.001	<0.001												
Providencia AA31 Dead	0.267	2.533	2.533	0.200											
	1.0	<0.001	<0.001	1.0											
K. oxytoca Live	−1.000	1.267	1.267	−1.067	−1.267										
	0.009	<0.001	<0.001	0.003	<0.001										
K. oxytoca Dead	−0.667	1.600	1.600	−0.733	−0.933	0.333									
	0.926	<0.001	<0.001	0.416	0.026	1.0									
Enterobacter 23 Live	1.867	4.133	4.133	1.800	1.600	2.867	2.533								
	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001								
Enterobacter 23 Dead	0.400	2.667	2.667	0.333	0.133	1.400	1.067	−1.467							
	1.0	<0.001	0.001	1.0	1.0	<0.001	0.003	<0.001							
Providencia 22 Live	−2.333	−0.067	−0.067	−2.400	−2.600	−1.333	−1.667	−4.200	−2.733						
	<0.001	1.0	1.0	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001						
Providencia 22 Dead	−1.400	0.867	0.867	−1.467	−1.667	−0.400	−0.733	−3.267	−1.800	0.933					
	<0.001	0.070	0.070	<0.001	<0.001	1.0	0.416	<0.001	<0.001	0.026					
Bacillus 139 Live	−2.533	−0.267	−0.267	−2.600	−2.800	−1.533	−1.867	−4.400	−2.933	−0.200	−1.133				
	<0.001	1.0	1.0	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.0	0.001				
Bacillus 139 Dead	−1.533	0.733	0.733	−1.600	−1.800	−0.53	−0.867	−3.400	−1.933	0.800	−0.133	1.000			
	<0.001	0.416	0.416	<0.001	<0.001	31.0	0.070	<0.001	<0.001	0.176	1.0	0.009			
Serratia 49 Live	−1.267	1.0	1.0	−1.333	−1.533	−0.267	−0.600	−3.133	−1.667	1.067	0.133	1.267	0.267		
	<0.001	0.009	0.009	<0.001	<0.001	1.0	1.0	<0.001	<0.001	0.003	1.0	<0.001	1.0		
Serratia 49 Dead	−1.067	1.200	1.200	−1.133	−1.333	−0.06	−0.400	−2.933	−1.467	1.267	0.333	1.467	0.467	0.20	−0.20
	0.003	<0.001	<0.001	0.001	<0.001	71.0	1.0	<0.001	<0.001	<0.001	1.0	<0.001	1.0	1.0	1.0

Table 5 Effect of the provision of bacteria in the host larval diet on egg-to-adult developmental duration of *Diachasmimorpha longicaudata* male offspring. Pairwise comparisons, first row of each cell: χ^2, second row: Bonferroni adjusted P ($\alpha = 0.003$)

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Descriptive statistics	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Mean (days)	20.15	19.27	19.29	19.42	19.53	20.59	20.62	20.23	20.11	20.37	20.37	20.42	20.15	19.33	19.32
SEM	0.079	0.078	0.084	0.081	0.075	0.079	0.082	0.082	0.079	0.081	0.095	0.064	0.075	0.072	0.078

Pairwise comparisons [Kaplan-Meier/log-rank (Mantel-Cox)]

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	52.75														
	<0.001														
Enterobacter AA26 Dead	47.31	0.07													
	<0.001	0.799													
Providencia AA31 Live	38.22	1.08	0.59												
	<0.001	0.299	0.443												
Providencia AA31 Dead	31.27	4.35	3.14	0.97											
	<0.001	0.037	0.077	0.325											
K. oxytoca Live	15.67	95.86	88.74	77.26	71.03										
	<0.001	<0.001	<0.001	<0.001	<0.001										
K. oxytoca Dead	17.13	93.09	86.35	75.70	30.13	0.06									
	<0.001	<0.001	<0.001	<0.001	<0.001	0.800									
Enterobacter 23 Live	0.12	47.80	43.06	36.47	26.87	11.37	12.98								
	0.732	<0.001	<0.001	<0.001	<0.001	0.001	<0.001								
Enterobacter 23 Dead	0.22	46.84	41.76	33.63	48.21	18.88	20.85	0.61							
	0.639	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.434							
Providencia 22 Live	3.59	70.89	64.83	55.04	43.77	4.18	4.89	2.03	5.45						
	0.058	<0.001	<0.001	<0.001	<0.001	0.041	0.027	0.155	0.020						
Providencia 22 Dead	4.31	64.07	58.74	49.39	57.59	2.32	2.71	2.51	6.10	0.10					
	0.038	<0.001	<0.001	<0.001	<0.001	0.128	0.100	0.113	0.014	0.750					
Bacillus 139 Live	4.46	82.85	75.89	65.51	32.55	4.79	5.99	2.72	6.82	0.001	0.12				
	0.035	<0.001	<0.001	<0.001	<0.001	0.029	0.014	0.099	0.009	0.977	0.732				
Bacillus 139 Dead	0.005	54.64	48.99	39.92	4.34	16.99	18.87	0.17	0.17	4.05	4.77	5.12			
	0.943	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.677	0.681	0.044	0.029	0.024			
Serratia 49 Live	53.27	0.002	0.043	0.99	3.66	95.19	92.71	51.98	48.44	71.63	62.61	85.82	56.15		
	<0.001	0.964	0.836	0.320	0.037	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
Serratia 49 Dead	51.90	0.04	0.005	0.69	71.03	94.56	92.23	50.47	47.09	70.08	61.33	84.07	54.69	0.04	0.04
	<0.001	0.846	0.946	0.405	0.056	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.837	0.837

Table 6 Effect of the provision of bacteria in the host larval diet on egg-to-adult developmental duration of *Diachasmimorpha longicaudata* female offspring. Pairwise comparisons, first row of each cell: χ^2, second row: Bonferroni adjusted P ($\alpha = 0.003$)

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Descriptive statistics	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Mean (days)	21.41	20.27	20.34	20.43	20.53	22.05	22.02	21.64	21.38	21.29	21.37	21.40	21.43	20.42	20.49
SEM	0.084	0.082	0.079	0.089	0.075	0.093	0.102	0.089	0.102	0.081	0.083	0.077	0.068	0.070	0.080

Pairwise comparisons [Kaplan-Meier/log-rank (Mantel-Cox)]

		Enterobacter AA26		*Providencia* AA31		*K. oxytoca*		*Enterobacter* 23		*Providencia* 22		*Bacillus* 139		*Serratia* 49	
Treatment	Control	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
Enterobacter AA26 Live	73.75														
	<0.001														
Enterobacter AA26 Dead	70.64	0.35													
	<0.001	0.552													
Providencia AA31 Live	55.49	1.54	0.51												
	<0.001	0.215	0.476												
Providencia AA31 Dead	52.32	3.08	1.51	0.24											
	<0.001	0.079	0.219	0.633											
K. oxytoca Live	31.32	131.8	129.2	108.3	104.6										
	<0.001	<0.001	<0.001	<0.001	<0.001										
K. oxytoca Dead	29.53	123.4	121.0	101.2	97.66	0.005									
	<0.001	<0.001	<0.001	<0.001	<0.001	0.945									
Enterobacter 23 Live	2.66	77.83	76.36	63.81	62.13	14.54	13.75								
	0.103	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001								
Enterobacter 23 Dead	0.01	68.26	65.51	51.5	48.52	28.98	27.37	2.17							
	0.921	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.141							
Providencia 22 Live	0.58	75.26	71.08	53.2	48.95	43.63	41.1	5.38	0.70						
	0.446	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.020	0.402						
Providencia 22 Dead	0.01	81.68	78.03	60.21	56.24	36.54	34.45	3.15	0.05	0.48					
	0.913	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.076	0.828	0.488					
Bacillus 139 Live	0.01	93.26	89.13	68.59	64.06	38.33	36.12	2.71	0.00	0.93	0.05				
	0.914	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.099	0.985	0.335	0.817				
Bacillus 139 Dead	0.03	90.62	86.72	67.75	63.67	38.37	36.15	3.80	0.09	0.41	0.01	0.12			
	0.855	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.051	0.761	0.523	0.924	0.729			
Serratia 49 Live	66.56	0.71	0.04	0.26	1.08	122.8	114.8	73.58	61.34	65.07	72.28	82.41	81.26		
	<0.001	0.400	0.808	0.613	0.299	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
Serratia 49 Dead	57.19	2.92	1.32	0.14	0.02	113.0	105.7	65.57	52.89	54.79	62.06	71.1	69.77	0.875	0.875
	<0.001	0.087	0.250	0.714	0.899	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.350	0.350

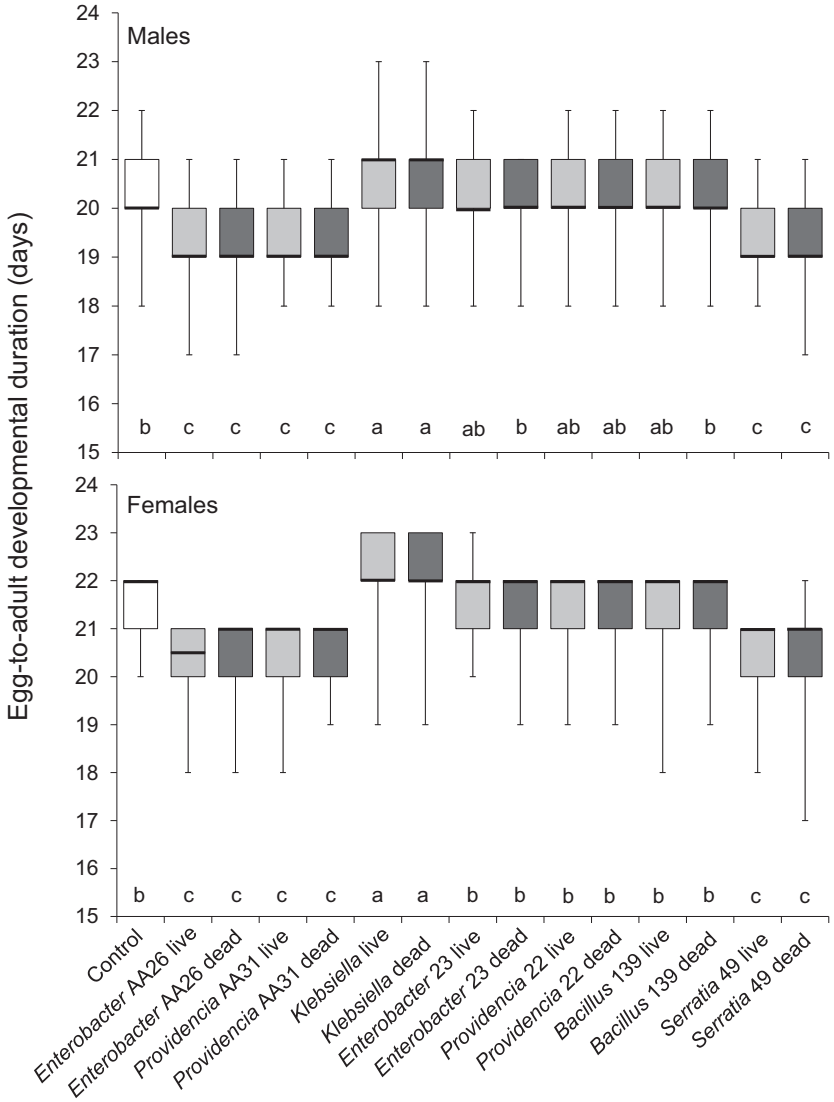

Figure 5 Effect of medfly larval diets enriched with LB medium (without bacteria; control), *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Klebsiella oxytoca*, *Enterobacter* sp. 23, *Providencia* sp. 22, *Bacillus* sp. 139, or *Serratia* sp. 49 on male (top) and female (bottom) *Diachasmimorpha longicaudata* egg-to-adult developmental duration (days). The top and bottom of the boxes represent the 25th and 75th percentiles, indicating inter-quartile range. The horizontal line within the box represents the median value. The whiskers indicate the highest and lowest observations and define the variability outside the inter-quartile range. Treatments marked with different letters on the x-axis cause a significant difference in egg-to-adult period (Mantel-Cox log-rank test: $P<0.003$).

important not only for the laboratory rearing but also for efficient parasitoid releases. Dead *Providencia* sp. 22 had no significant effect on the same life-history traits, which indicates that the effect is probably due to a positive effect of the live bacteria on the wasp. However, the effect of *Providencia* sp. 22 on olive fruit fly rearing is negative (Koskinioti et al., 2020); consequently, this bacterial isolate may be efficient only in parasitoid rearing systems that use medfly larvae as hosts for the parasitic wasp.

Live *Bacillus* sp. 139 treatment increased parasitoid fecundity, parasitism rate, sex ratio, and female production, whereas it had no effect on development rate of the progeny. Dead *Bacillus* sp. 139 bacteria had no significant effect on the *D. longicaudata* life-history traits except female production, which was increased compared to the control. These results demonstrate that live *Bacillus* sp. 139 has an overall positive effect on parasitoid rearing. *Bacillus* sp. 139 had an overall positive impact on the olive fruit fly

as well, in which it increased pupal weight (both live and autoclaved) and adult recovery (only autoclaved treatment) and reduced the time required for egg-to-adult development (Koskinioti et al., 2020). Therefore, it could be efficient in parasitoid rearing systems that use both medfly and olive fruit fly larvae as hosts. Again, the effect of rearing *D. longicaudata* on *Bacillus*-infected olive fruit fly larvae instead of medfly remains to be investigated.

Serratia sp. 49 (both live and autoclaved) had no effect on fecundity and parasitism rate but accelerated parasitoid emergence in both males and females. Live *Serratia* sp. 49 increased sex ratio and female production, whereas the autoclaved treatment did not affect sex ratio and increased female production per female to a lesser extent than the live treatment. Therefore, there is a positive effect of live *Serratia* sp. 49 bacteria on *D. longicaudata* rearing because they bias production towards females and induce faster production of parasitoids in general, whereby the total emergence rate of the parasitoids is not affected. On the other hand, the overall effect of live *Serratia* sp. 49 on olive fruit fly rearing was negative (Koskinioti et al., 2020) as it dramatically decreased olive fruit fly production. Hence, the use of *Serratia* sp. 49 appears efficient only in parasitoid rearing systems that use medfly larvae as hosts and the benefits are only related to the increased production of female progeny and the faster production of both male and female wasps.

Klebsiella oxytoca (live and autoclaved) delayed parasitoid emergence and had no effect on fecundity, parasitism rate, or sex ratio of the emerged wasps, whereas live *K. oxytoca* increased female production. Supplementing larval diet with *K. oxytoca* was also studied in both the medfly – in which it reduced the immature developmental duration but did not alter the production of medflies (Kyritsis et al., 2017) – and the olive fruit fly, in which it strongly reduced the production of *B. oleae* (Koskinioti et al., 2020). This indicates that, despite the positive effect that it has on medfly rearing, the use of *K. oxytoca* in a combined approach to improve parasitoid production in medfly or olive fruit fly rearing systems is not promising.

Live *Enterobacter* sp. 23 decreased parasitoid fecundity, parasitism rate, and female production, whereas the autoclaved treatment had no effect. Both live and autoclaved treatments had no effect on the sex ratio or the egg-to-adult developmental duration of the progeny. Our results with *Enterobacter* sp. 23 indicated that live treatment had a negative effect on parasitoid production whereas the autoclaved treatment had no effect on the same life-history traits. Therefore, *Enterobacter* sp. 23 is not a promising additive for the enhancement of *D. longicaudata* rearing.

Our results demonstrated that the positive effects of *Enterobacter* sp. AA26 (in female fecundity, parasitism rate, female production, and egg-to-adult developmental duration) and *Providencia* sp. AA31 (in egg-to-adult developmental duration) are similar for both the live and autoclaved treatments. This indicates that these isolates may function as nutrient sources that improve the growth and survival of larval hosts, and indirectly affect parasitoid production by offering more suitable hosts. On the other hand, the positive effect of *Bacillus* sp. 139 on female fecundity, parasitism rate, and female progeny production, and *Providencia* sp. 22 and *Serratia* sp. 49 on female progeny production is more evident for the live treatments of these isolates. This is an indication of a direct positive effect of the live bacteria on the parasitoid. These bacteria could be acquired by the parasitoid during development inside the fruit fly larva/pupa and then function as facultative endosymbionts of the parasitic wasp affecting various life-history traits. This hypothesis could be further explored by detecting the presence of bacteria in the parasitoid progeny that emerged from live-bacteria-fed flies, or feed adult wasps with the bacteria and see if this has an effect. Enhanced parasitoid fitness might be the result of potential bacteria-induced counteraction of the fruit fly defense system that facilitates parasitoid development inside the host.

Although encapsulation of wasp eggs has not been observed in *C. capitata*, as is the case in some *Bactrocera* spp. against the parasitoid *D. kraussii* (Ero et al., 2010), it is still possible that medfly larvae use another immune mechanism to defend themselves against parasitoids that is not yet known and this mechanism might be compromised by the presence of bacteria. Alternatively, increased parasitoid fitness and production might be the result of the increased size of the host. It has been observed that host body size affected the number of emerged *D. longicaudata* parasitoids: more parasitoids emerged from medium size hosts compared to small and large hosts (López et al., 2009). Similarly, the results of *Enterobacter* sp. AA26 indicated an increase in parasitism rate in our study and an increase of pupal weight in *C. capitata* (Augustinos et al., 2015).

Providencia sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 increased female proportion of parasitoid progeny. How the acquired host bacteria can alter the sex ratio of the wasps is unknown. At this point, we can only speculate about mechanisms. First, as the wasps have haplodiploid reproduction, with females arising from fertilized eggs and males from unfertilized eggs, the bacteria may directly affect the fertilization decision of the wasp. There are many symbionts known to bias the host sex ratio towards females (Bourtzis & Miller, 2008; Werren et al., 2008), but the bacteria tested here do not seem to belong to those groups. Also, it may be unlikely that the bacteria can

induce such an effect within a single generation after their acquisition by the wasp. A second possibility is that the bacteria somehow increase the survival of female progeny at the cost of male siblings. A third possible explanation is that the ovipositing female perceives the presence of the bacteria in the host as a cue of high host quality, which in turn induces her to produce more daughters. It is well known from parasitoid foraging literature that mothers can allocate daughters to high-quality hosts and sons to low-quality hosts (Charnov, 1982; King, 1987; Godfray, 1994). These possible effects of the bacteria on the wasp clearly warrant further investigation.

The negative effects of *Enterobacter* sp. 23 on fecundity and parasitism rate are also caused only when treated with live isolate. This is an indication that *Enterobacter* sp. 23 might be acquired by or interact with the parasitoid during its immature development inside the fruit fly larva. Interaction with the specific bacterial isolate could have a pathological effect on the wasp immature stages that potentially inhibits further development of the wasp inside the fruit fly host and subsequently leads to reduced parasitism rates. Studies in aphids have shown that aphid symbiotic bacteria play an important role in the defense of the host against its parasitic wasps (Oliver et al., 2003, 2014; Vorburger et al., 2010; Schmid et al., 2012). Similarly, several other studies have shown that the facultative endosymbiont *Spiroplasma* protects *Drosophila* spp. against parasitic wasps (Xie et al., 2010, 2011, 2014, 2015; Mateos et al., 2016; Paredes et al., 2016). Further investigation is required to prove whether *Enterobacter* sp. 23 could play a similar defensive role against the fruit fly parasitic wasps, such as by boosting the fly's immune system.

The fact that *D. longicaudata* can be reared on *C. capitata* is an advantage that overcomes the difficulties with the current rearing system of the olive fruit fly. Recent releases of *D. longicaudata* reared on *A. ludens* have been successful in suppressing *C. capitata* wild populations (Cancino et al., 2019), therefore, *D. longicaudata* reared on *C. capitata* might be used for *B. oleae* population suppression. Prior to any release, it first needs to be assessed whether *D. longicaudata* reared on *C. capitata* can parasitize olive fruit fly larvae. It is possible that these wasps will be less effective against *B. oleae* leading to unsuccessful parasitoid releases. Similar issues have been demonstrated by Canale & Benelli (2012) who proved that females with oviposition experience on a host species demonstrated higher preference for the same host species compared to others. In such case, it is crucial to further investigate the potential application of the bacteria that improved olive fruit fly rearing, as demonstrated by Koskinioti et al. (2020), to improve parasitoid rearing using the olive fruit fly as the rearing host, instead of the medfly. Also the effect of bacteria-enriched larval diet on life-history traits of the medfly host requires further investigation.

The Mediterranean fruit fly was selected as the host in our study because it is the most widely used tephritid in SIT applications and is currently used in 15 mass-rearing facilities involved in medfly control programs using SIT (DIR-SIT, 2019). Therefore, it would be more cost-efficient to combine parasitoid production with a *C. capitata* rearing facility, as *B. oleae* mass rearing is currently inefficient.

In conclusion, ours is the first study to investigate the potential effect of fruit fly gut symbionts on the efficacy of parasitoid wasp rearing systems. Our results demonstrate that use of *Enterobacter* sp. AA26, *Providencia* sp. AA31, *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 as supplements/probiotics of host larval diets is a promising strategy for the improvement of the current *D. longicaudata* rearing system and can also serve as an example for the improvement of laboratory rearing of other parasitic wasps. However, the application of live bacteria under laboratory conditions raises concerns regarding biosafety and biosecurity. Inactivated bacterial forms may actually be more easily accepted for use in mass-rearing facilities, but this would exclude the beneficial aspects of *Providencia* sp. 22, *Bacillus* sp. 139, and *Serratia* sp. 49 probiotic diets. *Enterobacter* sp. AA26 and *Providencia* sp. AA31 could still be used as additives in their inactivated/dead form. In general, an increase in female fecundity, parasitism rate, female progeny production, and reduction in the time required for egg-to-adult development, are traits that would lead to increased parasitoid production. Positive effects of the gut bacterial isolates on parasitoid production combined with positive effects on fruit fly larvae production would further enhance the efficacy of the *D. longicaudata* rearing system. This, in turn, might contribute to an efficient IPM program for harmful fruit flies. Our study mainly focused on improvement of parasitoid rearing efficiency (i.e., the quantity of the parasitoids). Further investigation of the effect of beneficial bacterial isolates on life-history traits related to the fitness of the parasitoids after release in the field (i.e., their quality) – such as flight ability, dispersal capacity, and survival – could strengthen the case for their applicability to the improvement of IPM programs.

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